REGULATION OF VEGETATIVE AND GENERATIVE REPRODUCTION IN THE WOODLAND STRAWBERRY

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Abstract

Unlike annual plants, perennials have repeated cycling between the vegetative and generative stages. Studying the balance between these two phases would enable breeders to produce higher quality crops. The woodland strawberry is used as a model to study developmental patterns in perennials because it has a wide geographical distribution, a small sequenced genome, and a number of available natural mutants, which provide excellent resources for physiological, molecular and genetic studies. This thesis investigated the genetic and environmental coordination of shoot apical meristem (SAM) and axillary meristem (AXM) fates in woodland strawberry. In woodland strawberry, SAM forms an inflorescence after flower induction, whereas AXMs can differentiate either into runners or branch crowns that are able to form additional inflorescences.

Genetic mapping and the experiments using transgenic lines and natural accessions with contrasting environmental responses showed that a number of genes regulated the balance between vegetative and generative development in woodland strawberry. In general, cool temperature or short days (SD) induced flowering and promoted AXM differentiation to branch crowns, while warm temperature and long days (LD) promoted runner formation. High levels of *FvTERMINAL FLOWER1* (*FvTFL1*) expression in *FvTFL1* overexpression lines and NOR1 accession inhibited flowering at temperatures of 10-22°C in both SD and LD, but the environmental control of AXM fate was not affected in these plants indicating that environment influenced AXM differentiation irrespective of flowering. In the seasonal flowering genotype, *FvSUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*FvSOC1*) was observed to quantitatively increase runner formation.

The photoperiodic control of flowering and AXM fate was studied in more detail using *FvCONSTANS* (*FvCO*) and *FvFLOWERING LOCUS T1* (*FvFT1*) transgenic lines. These studies

showed that FvCO controls the expression of *FvFT1,* and they both have a major role in the control of the balance between the vegetative and generative development in SD and LD.

Genetic mapping studies under differing environments identified five QTLs that, together, explained about half of the observed flowering time variance in the mapping population, and two additional QTLs were identified for the number of branch crowns explaining about 20% of variance. The flowering time QTL on LG6 colocalized with *FvTFL1,* and one of the QTL regions on LG4 that controlled both flowering time and AXM fate was close to the *PFRU*, a previously identified locus in the commercial strawberry. Among the previously unknown loci, two flowering time QTLs on LG7 colocalized with putative flowering time genes *FvEARLY FLOWERING 6 (FvELF6)* and *FvCENTRORADIALIS1 (FvCEN1)*, a homolog of *FvTFL1.* Furthermore, a gene encoding TCP transcription factor and a homolog of *DORMANCY ASSOCIATED MADS BOX (DAM)* were identified as candidate genes in QTL regions controlling AXM fate on LG4 and LG5, respectively. This study shed new light into the genetic and environmental control of AXM and SAM fates providing new means to control the balance between vegetative and generative reproduction under different environmental conditions.

Contents

List of original publications

The thesis is based on two publications and one manuscript which are referred to in the text in Roman numerals (I – III):

I. **Samad, S.**, Koskela, E., Hytönen, T. 2017. "Regulation of axillary meristem fate in woodland strawberry." [Manuscript]

II. Kurokura, T., **Samad, S.**, Koskela, E., Mouhu, K., Hytönen, T. 2017. "Fragaria vesca CONSTANS controls photoperiodic flowering and vegetative development". *J. Exp. Bot*. 68(17): 4839–4850, doi.org/10.1093/jxb/erx301

III. **Samad S.**, Kurokura, T., Koskela, E., Toivainen, T., Patel, V., Mouhu, K., Sargent D.J., Hytönen, T. 2017. "Additive QTLs on three chromosomes control flowering time in woodland strawberry. (*Fragaria vesca* L.)". *Hort. Res*. 4, 17020–, doi:10.1038/hortres.2017.20

Authorship statement

In (I), the experiments were planned and designed by SS, EK and TH. SS had the main responsibility in phenotyping observations and statistical analysis. EK coordinated the study and participated with phenotyping and statistical analysis. The manuscript was written by SS, EK and TH.

In (II), the experiment was designed by TH and TK. SS took part in the phenotypic observations with EK and TK. RT-qPCR were performed by SS and TK. KM generated the transgenic lines while TK was involved in phylogenetic analysis. TH supervised the study. TH together with TK wrote the final manuscript.

In (III), SS and TK shared first authorship of the manuscript. TK and TH were involved in experimental design and phenotypic observations. TK also did candidate searches and analyzed the data. SS carried out the genetic mapping, designed and optimizing primers, prepared the samples for genotyping-by-sequencing (GBS) analysis. DS participated with QTL mapping and GBS analysis. The F1 cross, DNA extraction and hereditary analysis was conducted by KH. The F2 mapping population was developed by EK. EK was also involved in marker development and phenotypic observations. VP was involved with whole genome sequence analysis. TT analyzed GBS data and SNP mining. TH supervised the work and wrote the final manuscript with SS and TK.

1 INTRODUCTION

Plants are sessile organisms and thus, the reproductive success of plants relies on consistently observing and responding to their environment (Bernier and Périlleux, 2005). Therefore, plants have developed complex molecular networks and systems to monitor and integrate various internal and external cues such as temperature, photoperiod (Tan and Swain, 2006) and also hormones (Levy and Dean, 1998).

Plant growth is dependent on the formation of undifferentiated meristematic cells that form new organs. The shoot apical meristem (SAM) is such a group of cells that is found at the shoot apex. The SAMs in the annuals and perennials behave differently when plant transits to generative stage (Battey, 2000; Thomas *et al.*, 2000). In annual plants the commitment to generative phase is permanent and flowering followed by senescence is the last stage in its life cycle. In these plants, the SAMs in all shoots enter generative phase at the same time, known as monocarpic growth habit. Polycarpic perennial plants have reiterative vegetative and generative stage and may return to vegetative stage after flowering (seasonal flowering) or may continuously flower after induction (perpetual flowering) (Brown and Wareing, 1965; Darrow, 1966). For a plant to be termed perennial, at least one meristem remains vegetative for the next season (Battey, 2000; Thomas *et al.*, 2000).

Most perennial plants have economical values and produce edible fruits, which make up a significant part of the daily diets. Therefore, understanding the balance between vegetative and generative phase and the underlying mechanisms is a key ingredient in breeding higher quality crops. With the climate changing becoming more unpredictable, to acquire this knowledge has become even more important because temperature has an impact on flowering and by extension on the yield.

1.1 Vegetative stage

The vegetative stage of a plant begins from germination until the onset of sexual reproduction. During this stage, plants produce vegetative structures, increase in size and mass while at the same time remain insensitive to floral induction. The primary shoot axis of the plant is formed during embryonic development and is defined by the shoot apical meristem (SAM) (Sussex, 1989). In dicots such as *Arabidopsis*, the SAM is centralized that is in between the cotyledons. Whereas in monocots like rice and maize, the SAM forms at the base of a single cotyledon. The sides of the SAM produce leaf primordia and a small region of stem cells grow between the SAM and the primordia known as the axillary meristems (AXM). New axes grow and produce additional AXM, which are reiterated as modules or phytomer. Each phytomer is composed of a number of vegetative structures such as stem, nodes bearing leaves or leaf-like structures, internode and AXM. The various forms of architecture in plants are influenced by the number of phytomers, their production and the relationship between the different components (Sussex, 1989; Bennett and Leyser, 2006), which coordinates the initiation, differentiation and development of different organs from branches to inflorescence (Costes *et al.*, 2014; Janssen *et al.*, 2014).

1.1.1 Vegetative development

The meristematic tissue is very flexible and is organized throughout the lifecycle of the plant; it can remain dormant or form vegetative structures such as leaves and branches or it can become determinate and form inflorescence (Kerstetter and Hake, 1997). The fate of the meristem can also be influenced by environmental cues, such as day length and temperature. This ensures that flowering only occurs during favorable conditions (Griffiths and Halliday, 2011).

In some plants, the vegetative growth can further be divided into juvenile and adult vegetative stages. In tree species, the juvenile stage can extend for a number of years before the plant becomes matured. During the juvenile stage, the plant lacks reproductive competence even during favorable environment while during the adult phase, the plant can transition into generative stage if induced (Sussex, 1989). The AXM can record phase changes because lateral buds during the vegetative phase are vegetative and the same is true for juvenile and adult phases, where AXM under each stage produces phase specific shoots. For example the annual model, *Arabidopsis* produces small round shaped leaves during the juvenile phase, larger narrower leaves during the adult stages and branches after floral transition. (Grbić and Bleecker, 2000; Poethig, 2003). In the pea, the AXM develops at the nodes along the stem, while in *Arabidopsis* there is a delay in the formation of AXM and thus some nodes may lack AXM.

1.1.2 Genetic control of vegetative development

Several genetic pathways regulate the vegetative state of the plant and one of the main group fall under the TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1 (TCP). The TCP transcription factors are a small family of transcription factors specific to plants that play vital role in regulation and proliferation of plant growth and development (Kieffer *et al.*, 2011). TCP factors have been studied in many plants and have shown to affect branching, leaf and flower development and also development through the hormone pathways (Aguilar-Martínez *et al.*, 2007; Kieffer *et al.*, 2011). TCP transcription factors have two gene clades, THE CIN-like clade is involved in lateral organs and the CYC/TB1 clade that controls the AXM development (Wei *et al.*, 2016). *TB1* in maize inhibits branching and a single homolog was found to be conserved in monocots. While in dicots like *Arabidopsis,* multiple orthologs have been identified such as *BRANCHED1* (*BRC1*) and

BRANCHED2 (*BRC2*) that inhibit AXM outgrowth (Doebley *et al.*, 1995; Aguilar-Martínez *et al.*, 2007; Kieffer *et al.*, 2011; Kebrom *et al.*, 2013).

Plant hormones are mobile molecules that regulate plant development in minute concentrations and bind to specific receptor proteins. Thus, the spatial and temporal concentration of hormones is important for them to interact with receptors (Frébort *et al.*, 2011). Auxin and its role has been known in a phenomena known as ”apical dominance”, which is the predominant growth of the main axil while inhibiting axillary bud outgrowth. (Thimann and Skoog, 1933). Cytokinin (CK) hormones promote cell division and axillary bud outgrowth; thus, is antagonistic to auxin (Werner *et al.*, 2001). A study in pea (*Pisum sativum* L.) also proposed that auxin inhibits CK biosynthesis in the stem nodal (Tanaka *et al.*, 2006).

Gibberellins (GA) also promote cell division, elongation (Mutasa-Göttgens and Hedden, 2009), stimulate seed germination and control phase changes such as floral transition (Gupta and Chakrabarty, 2014). GA is regulated by endogenous cues such as auxin and environmental signals such as light and temperature. These cues can directly influence GA metabolism or alter the accumulation of growth repressors such as DELLA proteins therefore, affecting GA function (Sun, 2008). GA promotes plant growth by regulating DELLA proteins that repress GA activity by directly binding to the promoters of GA-regulated genes (Mutasa-Göttgens and Hedden, 2009). In *Arabidopsis,* GA deficient mutants displayed a dwarf phenotype and gibberellin-insensitive (GAI) mutant abundantly produced axillary shoots (Koornneef and van der Veen, 1980; Wilson and Somerville, 1995); conversely in the woody perennial *Jatropha curcas*, GA is needed to facilitate CK to promote lateral bud outgrowth through putative homologs of BRC1 and BRC2 (Ni *et al.*, 2015).

1.2 Generative reproduction

The transition from vegetative to generative phase is a vital survival strategy in plants. This typically occurs in the SAM and is coordinated by both internal and external signals. At adult phase, a certain endogenous balance is attained, favorable signals such as photoperiod and temperature are perceived in the leaves and a mobile signal from the leaves is transmitted the SAM (Wellensiek, 1964; Aukerman and Amasino, 1998). The signal induces the plant to converse the vegetative SAM to an inflorescent meristem that forms the flower primordia, forming the precursor for flowers and flowering shoots and the process is called floral transition (Kerstetter and Hake, 1997).

After attaining maturity, the plant cycles between the vegetative and generative stages by forming vegetative or generative structures from the AXM. In *Arabis alpina*, the cycling between the two stages is controlled by *PERPERTUAL FLOWERING 1 (PEP1)*, which is an orthologue of *FLOWERING LOCUS C (FLC)*, a floral repressor in *Arabidopsis*. *PEP1* is a MADS-box transcription factor that is epigenetically regulated by histone modifications (Wang *et al.*, 2009). In *Arabidopsis*, after the terminal meristem becomes generative, the lateral vegetative meristems also produce inflorescence branches. However the opposite is also true in some perennial species like the tomato, where the lateral meristems form inflorescence while the primary meristem remain vegetative (Poethig R. S., 1990). In Poplar, the terminal shoot remains indeterminate through the lifecycle but matured trees develop AXM after winter dormancy (Mohamed *et al.*, 2010). Since the axillary meristems can produce either the vegetative or generative structures, there is a balance between the two developmental stages and it is hypothesized that vegetative structures prevent flowering (Geber, 1990; Bonser and Aarssen, 2006).

Molecular regulation of flowering has been studied in details in *Arabidopsis*, which has several genetic pathways to flower. The main pathways to flower are photoperiod, temperature,

autonomous, GA and vernalization pathways. These pathways converge at genes known as floral integrators, which include floral promoters FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) (Blázquez *et al.*, 1998; Nilsson *et al.*, 1998; Hayama *et al.*, 2004). These integrators are then responsible for the activation of the meristem identity genes such as *APETALA1* (AP1) (Kaufmann *et al.*, 2010), which initiate flowering.

1.2.1 The photoperiodic pathway

One of the most important environmental signal for perennial plants is photoperiod, which is the measure of daylength. Photoperiod is the only accurate seasonal cue because it follows the same pattern every year (Andrés and Coupland, 2012). Garner and Allard in 1920 were the first to demonstrate how photoperiod could manipulate flowering and classified plants according to their reaction to daylength. Long day (LD) plants flower when they are exposed to photoperiod which exceeds a certain threshold. While short day (SD) plants flower when the length of the night exceeds a critical threshold (Jarillo *et al.*, 2008; Fujiwara *et al.*, 2008; Blackman, 2017). Day neutral plants on the other hand, are photoperiod insensitive and flower both in LD and SD conditions (Sønsteby and Heide, 2007).

The photoperiodic pathway (Figure 1) is one of the most studied flowering pathways, which has been characterized in the LD *Arabidopsis*. In *Arabidopsis*, the photoperiodic control of flowering time is connected to the circadian clock, which regulates the oscillation of output genes and synchronizes it to approximately 24 hrs in order to match the daily light hours (Fujiwara *et al.*, 2008). In 1936, Bünning acknowledged that there was a relationship between light and the circadian clock that initiated flowering. This theory was later developed and termed external

coincidence by Pittendrigh who stated that the circadian clock cycles and the plant's sensitivity to light changes in different phases of the clock cycle (Pittendrigh and Minis, 1964).

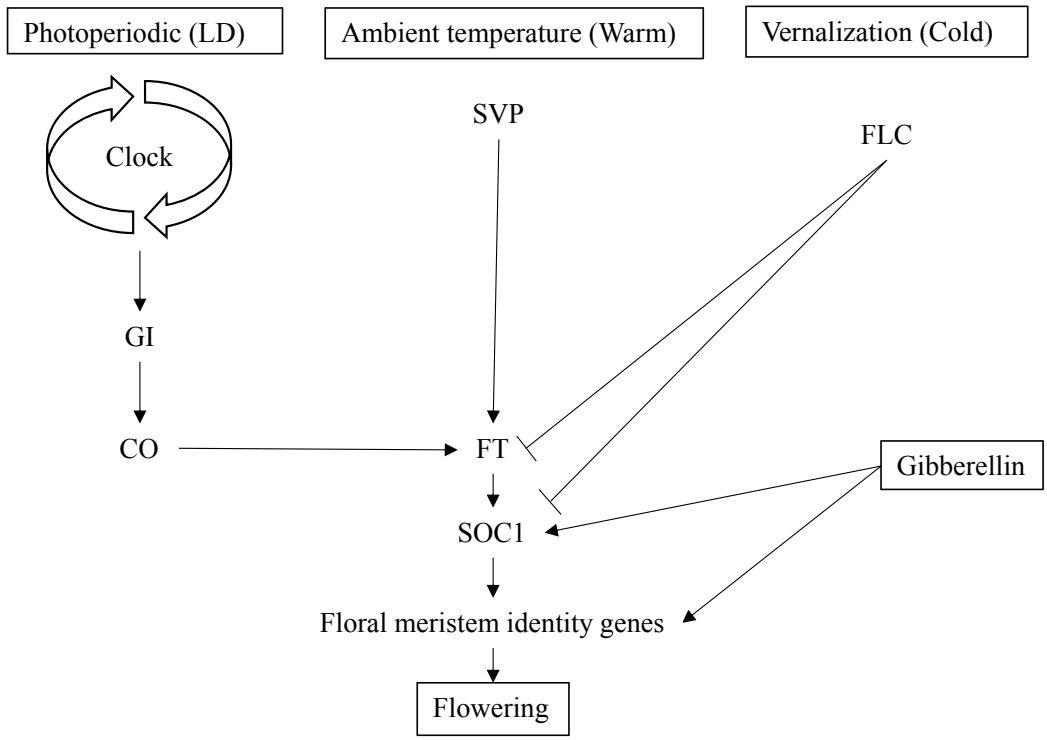

Figure 1 A simplified model of the main flowering pathways in Arabidopsis. The arrows indicate activation while the bars represent repression.

One of the key players of the pathway is *CONSTANS* (CO), which encodes a zinc finger transcription factor and is regulated both at transcriptional as well as at post-transcriptional levels (Putterill *et al.*, 1995). *CO* mRNA builds up in the leaves and the protein functions as an activator of *FT* during LDs but not SDs (Yanovsky and Kay, 2002; Sawa and Kay, 2011). This is because during LDs, *CO* expression coincides with light conditions when the CO protein is stabilized

(Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). Under LD, CYCLING DOF FACTOR1 (CDF1) peaks first during the day and binds to the promoter of *CO* to represses its expression. GIGANTEA (GI) accumulates next and forms a complex with CDF1, which also prevents *CO* accumulation. Finally in the late afternoon, FLAVIN BINDING KELCH REPEAT F-BOX1 (FKF1) reaches its peak and forms a complex with GI to degrade the CDF proteins, allowing the accumulation of *CO* mRNA (Sawa and Kay, 2011).

Furthermore, a stable CO protein is only formed in the presence of light in the afternoon in LDs. The CO protein is degraded in the dark by proteasome activity of an E3 ubiquitin ligase encoded by *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*). This regulates the CO protein levels, enabling it to accumulate only when plant is exposed to long photoperiod in order to induce flowering by activating FT (Corbesier *et al.*, 2007; Li, 2011).

Once activated by CO, the FT protein moves to the SAM, where it binds to form a complex between 14-3-3 protein and *FLOWERING LOCUS D* (*FD*) (Kobayashi *et al.*, 1999; Abe *et al.*, 2005). The complex then stimulates flowering by up regulating floral identity genes *AP1, LEAFY* (*LFY*) and *FRUITFULL* (*FUL*) genes (Albani and Coupland, 2010).

FT is the universal floral inducing signal in many plants and belongs to the member of the phosphatidylethanolamine-binding protein (PEBP) family. The PEBP family has diverse functions involving signaling pathways, growth and differentiation and contain both floral promoters and repressors (Hanzawa *et al.*, 2005).

The PEBP family also consists of another important gene, *TERMINAL FLOWER1* (*TFL1*) (Shannon and Meeks-Wagner, 1991). *TFL1* is a floral repressor and has an antagonistic function to that of *FT* (Hanzawa *et al.*, 2005). Low expression of *TFL1* is found in the lower parts of the apical meristem during the vegetative stage and the protein moves to the apex to repress flowering.

In *Arabidopsis*, *TFL1* is up-regulated after floral induction to continue to maintain the indeterminate inflorescence meristem (Pidkowich *et al.*, 1999). Mutations in the *Arabidopsis TFL1* can reduce the length of the vegetative stage by converting the *Arabidopsis* inflorescence to determinate. While in both primary and lateral shoots, the vegetative phase is extended by overexpressing *TFL1* (Alvarez, 1992).

SOC1 codes for a MADS box transcriptional factor (Lee and Lee, 2010), which is activated by the interaction of *FT* and *FD* and it has several repressors in the apex (Lee *et al.*, 2007; Immink *et al.*, 2012). *SOC1* expression in SAM is one of the earliest markers involved in the floral transition pathway (Albani and Coupland, 2010). *AP1* is a floral identity gene that codes for a MADS box transcription factor. *AP1* is involved in the floral differentiation (Abe *et al.*, 2005), and its activation indicates the end of the floral transition and the start of the development of flowers (Wellmer and Riechmann, 2010; Gómez-Ariza *et al.*, 2015). Kaufmann (2010) speculated that *AP1* directly or indirectly reduces the expression of *TFL1* by binding to the 3' end of the gene.

Although it is said that the flowering pathways are conserved across most species and that most perennials have similar genes to that of *Arabidopsis*; however, some of these play a different function in perennial plants or may have two or more homologues or paralogues playing the same function. One such example is the perennial poplar (*Populus spp*.), which has two *FT* paralogs (Ruiz-García *et al.*, 1997), *FT1* and *FT2*, that show different temporal expression patterns and functions. *FT1* initiates generative development upon perceiving winter, while *FT2* promotes vegetative growth as the days get longer and warmer (Hsu *et al.*, 2011).

1.2.2 The temperature pathway

Temperature also has a strong role in influencing flowering through the vernalization (Sung and Amasino, 2005) and ambient temperature pathways. Vernalization is a cold requirement by certain plants to induce flowering while small fluctuations in the surrounding temperature are controlled through the ambient temperature pathway (Wigge, 2013).

Natural winter annual accession of *Arabidopsis* requires winter chilling period prior to flowering and functional alleles of two genes, *FRIGIDA* (FRI) and *FLC*. FRI activates FLC and the protein binds to the gene sites of *FT* and *SOC1,* inhibiting flowering. Vernalization regulates this repression through epigenetic factors such as histone modifications by repressing FLC expression and in annuals, this downregulation is stable even after plants are moved to warm conditions (Napp-Zinn, 1987; Michaels and Amasino, 2001; Amasino, 2004; He and Amasino, 2005).

The key factor in the vernalization pathway is the regulation of FLC. This requires other factors such as VERNALIZATION INSENSITIVE 3 (VIN3), which is a plant homeodomain protein. VIN3 expression increases with the duration of the cold exposure, which correlates to the degree of *FLC* inhibition (Sung and Amasino, 2004). During vernalization, *FLC* levels drops through the increase in trimethylation at the lysine 27 residue of the histone 3 tail (H3K27me3) at the FLC locus by the interaction of VIN3 and POLYCOMB REPRESSION COMPLEX2 (PRC2) and this enables flowering during subsequent LD (Bastow *et al.*, 2004; Jeong *et al.*, 2009).

In addition to that, the ambient temperature pathway also regulates *FT* and *SOC1* (Balasubramanian *et al.*, 2006). The temperature regulation in flowering has also been studied in the annual *Arabidopsis,* where warm temperature results in early flowering, while cooler temperatures lead to delayed flowering responses (Balasubramanian *et al.*, 2006; Jarillo and

Piñeiro, 2011). On the other hand, an increase in temperature in *Boechera stricta*, a perennial relative of *Arabidopsis* delays flowering (Anderson *et al.*, 2011).

Further research has also shown an epigenetic mode of regulation (Kumar and Wigge, 2010; Ito *et al.*, 2012). Recent research in *Arabidopsis* has shown that there is epigenetic regulation involved in this pathway. One such study shows that production of FT is influenced through changes in the chromatin by histone complex. Under low temperatures, the FT promoter is restricted via chromatin compression by H2A.Z, a variant of the normal H2A. However at higher temperature, H2A.Z is removed, enabling transcription factors to access the FT promoter (Kumar and Wigge, 2010). One such transcription factor activated at high temperature is PHYTOCHROME INTERACTING FACTOR4 (PIF4), a bHLH transcription factor that binds to the FT promoter and induces early flowering under SDs (Kumar *et al.*, 2012).

In *Arabidopsis* there are five FLC-related loci named *MADS AFFECTING FLOWERING* genes (MAF 1–5), which are involved in the temperature regulation of flowering. MAF1 has also been named FLM and acts as a flowering repressor (Ratcliffe *et al.*, 2001). Under LD, flowering under high temperature is controlled through the interaction of MADS box transcription factors SHORT VEGETATIVE PHASE (SVP) and FLM. The floral repressor SVP, is degraded under high temperatures(Lee *et al.*, 2013), while FLM was thought to undergo temperature –dependent alternative splicing to regulate the flowering responses under the differing temperatures. It was suggested that both variants bind and compete in an antagonistic manner to form a heterodimer with SVP. However, the balance was shifted towards the floral promoter FLM-δ, under high temperatures and towards FLM-β, the repressor form under low temperatures (Posé *et al.*, 2013). Under low temperatures, the SVP-SVP and SVP-FLM-β complexes delay flowering by downregulating SOC1 and FT. Whereas at higher temperatures, the SVP-FLM-δ complex is not

able to repress flowering due to the inability to bind to FT and SOC1 promoters (Lee *et al.*, 2013; Posé *et al.*, 2013). Recent research using CRISPR/Cas9 to create specific exon deleted lines showed that the role of FLM-δ in floral regulation is negligible (Capovilla *et al.*, 2017).

1.3 Strawberry, an economically important berry

One of the most commercially important soft fruit is strawberry (*Fragaria*), which has a worldwide market and has been included in The International Treaty on Plant Genetic Resources, Annex 1 (Hummer *et al.*, 2011). Hence, examining traits which are important to increase and produce a better yield has become the focus for many studies. It was recorded that in 2014, the worldwide production of strawberries exceeded 8.1 million metric tons, out of which Europe produced about 20%. China and USA were the top producers, producing 3.1 million and 1.3 million tons respectively (FAO Statistics Division 2016, 2016).

Strawberry is a perennial plant of the genus *Fagaria* of the Rosaceae family, subfamily Rosoideae (Staudt, 2006). The Rosaceae family consists of 3000 species in about 90 genera (Illa *et al.*, 2011) and consists of fruit trees like *Malus* (apple) and *Pyrus* (pear); stone fruits from the *Prunus* such as peach and cherry; berries such as *Fragaria* (strawberry) and ornamentals such as Rosa (rose) (Dirlewanger *et al.*, 2002; Cabrera *et al.*, 2009; Shulaev *et al.*, 2011; Longhi *et al.*, 2014). The members of the family are both phenotypically as well as genetically diverse with differences in plant habit, fruit type and also in chromosome numbers which range from x = 7 to x = 17 (Illa *et al.*, 2011).

The *Fragaria* genus has 27 known taxa, which range from the diploid to decaploid and it also has a number of natural hybrids (Njuguna *et al.*, 2013). Many of the diploids and tetraploids are found in Asia, near the Sea of Japan and the Sino-Himalayan region (Darrow, 1966; Hummer *et al.*, 2011; Njuguna *et al.*, 2011; Liston *et al.*, 2014).

The cultivated strawberry *F.* × *ananassa ssp. ananassa* Duchesne ex Rozier is an octoploid species (Njuguna *et al.*, 2011). The history of the cultivated strawberry is traced to Europe during the mid-1700s, where a coincidental cross between octoploid species *F. chiloensis* (Mill.) and *F. virginiana*

(Duch.) took place forming an allo-octoploid (2n =8x = 56) species called *Fragaria* × *ananassa* Duch., which is now commercialized (Rousseau-Gueutin *et al.*, 2008; Gil-Ariza *et al.*, 2009; Bassil *et al.*, 2015; Mahoney *et al.*, 2016).

The family is a vital plant family in the temperate region and the fruits have been an important source of food since ancient times and eaten in various forms from jams to juices and this gives rise to many combinations of flavours and textures thus, a higher consumer choice (Dirlewanger *et al.*, 2002). Over time, selections and domestication of the plants have given rise to large fleshy fruits making them different from their wild relatives.

1.4 The woodland strawberry, a perennial model

The diploid *Fragaria vesca* has been actively researched on because it is one of the progenitors of the commercial octoploid strawberry (Gil-Ariza *et al.*, 2006). It has been proposed as a model plant for molecular analysis of perennial crops as well as that of the Rosaceae family, because it is easier to study due to its small diploid genome of 219 Mbp (Ruiz-Rojas *et al.*, 2010; Shulaev *et al.*, 2011). Moreover, it is easy to grow and propagate from seed, is quick to reproduce because it has a short generation time and moreover, it is easily transformed by Agrobacterium tumefaciens (Oosumi *et al.*, 2006; Ruiz-Rojas *et al.*, 2010). *Fragaria* has diverse phenotypes (Sargent *et al.*, 2004*b*), genotypes (Hummer *et al.*, 2011) and a vast distribution spreading across boreal Eurasia, North America and introduced into Japan and the Hawaiian Archipelagos (Hummer *et al.*, 2011; Njuguna *et al.*, 2011), which makes it a unique model that is easily available in different environmental conditions. It also has naturally occurring mutants to study early flowering and also runnerless accessions. Furthermore, it also has genotypes which induce flowering in short days (SD) such as *F. vesca* L. and also long days (LD) for example Fragaria *vesca semperflorens* Hawaii and 'Baron Solemacher' (Darrow and Waldo, 1932; Mouhu *et al.*, 2009) and it can form viable hybrids from crosses between different species within its taxa to produce new varieties (Schulze *et al.*, 2011).

1.5 Strawberry physiology

In *Fragaria*, the stem is a thick rootstock with short internodes and this together with the terminal bud is called a crown. *Fragaria* produces trifoliate leaves arranged in a rosette and each leaf axil has an axillary bud that can differentiate into runner (Figure 2), which is a long shoot made of two long internodes and ending with a leaf rosette (daughter plant) or can form a new leaf rosette known as branch crown (Figure 2). Daughter plants are genetically identical to the mother plants and can be utilized for vegetative reproduction (Hytönen and Elomaa, 2011).

Figure 2 Picture of woodland strawberry under vegetative condition. Picture on the left shows (1) a runner, (2) the daughter plant (3) leaf. On the right (4) circulates a branch crown.

Vegetative development is vital for plants to continue growing and in strawberries this also allows asexual propagation through runners. The vegetative development is perceived by growth rate, which can be measured by observing the leaf size, petiole length and increase in runner production (Durner and Poling, 1985).

Runnering and petiole length is regulated through environmental conditions such as temperature, photoperiod, hormones, nutrient and their interactions. In most accessions like the SD *Fragaria vesca* accession, LD and high temperature increases the rate of AXM producing runners, while SD promote branch crowns. Crown branching indirectly increases cropping potential by increasing the number of meristems that can transition to inflorescence meristems and produce flowers. The effect of the environment on the plant varies due to the vast diversity within the genus (Pure *et al.*, 1973; Heide *et al.*, 2013).

Once the plant is induced to flower, AXMs produces branch crowns and the apical meristem terminates with a primary flower and this is proceeded with two lateral branches which each terminates with a secondary flower. Each primary branch gives rise to two secondary branches and each terminates with a tertiary flower (Guttridge, 1985).

There are four stages to flowering namely, induction, initiation, differentiation and development. Induction occurs in the leaf, when FT relays the signal that causes the formation of a floral bud in the meristem while initiation summarizes the physiological and morphological changes happening in the meristem post induction. This is followed by the formation of floral organs known as the differentiation stage while the production of flowers is the final developmental step (Durner and Poling, 1985).

Floral initiation occurs in the shoot apex of the main crown. The inflorescence are formed from terminal apical meristems, after which the control of crown extension is taken over by the uppermost lateral bud, which becomes dominant over other lateral crowns (Heide *et al.*, 2013).

In both the garden strawberry and woodland strawberry, flowering is controlled by a complex photoperiod x temperature interaction. Flowering occurs independently of photoperiod at low temperatures, at SD (typically less than 14 hrs of day light) during intermediate temperatures

(between 14℃ – 20 ℃) and is repressed during high temperatures (>21℃). These critical temperatures and daylength vary based on accessions and cultivars (Heide and Sønsteby, 2007).

The alpine strawberry *F. vesca var. semperflorens* Duch. is unique as it requires LDs at intermediate (15-21℃) and high temperatures (> 26℃) to flower (Darrow, 1936; Sønsteby and Heide, 2007), while at low temperatures (< 9℃), photoperiod has a quantitative effect on flowering in some accessions. Flowering was also reported to be suppressed in these genotypes under SD with increasing temperature (Sønsteby and Heide, 2008; Heide *et al.*, 2013).

In *Fragaria*, flowering is antagonistic to runner formation because when induced to flower, the runner production reduces in both genotypes. The perpetual flowering genotypes have poor runner production probably because these are induced to flower at an early age (Sønsteby and Heide, 2007). Both traits can be manipulated by environmental cues such as photoperiod and temperature and their interaction thus, allowing for both genetic and phenotypic studies of growth and development as a model plant (Sønsteby *et al.*, 2013).

1.6 Genetic and molecular studies in strawberry

New varieties of strawberry are frequently being introduced into the market and stable methods to identify them is essential for breeders especially when these varieties are clonally propagated. In addition to that, genetic markers are also used for exploring strawberry genetic variation (Sargent *et al.*, 2004*b*; Brunings *et al.*, 2010), diversity (Gil-Ariza *et al.*, 2006; Njuguna *et al.*, 2011) and form molecular maps (Weebadde *et al.*, 2008; Gaston *et al.*, 2013; Castro *et al.*, 2015; Honjo *et al.*, 2016). Due to the synteny in the *Rosaceae* family, markers (Vilanova *et al.*, 2008; Zorrilla-fontanesi *et al.*, 2011; Gar *et al.*, 2011; Longhi *et al.*, 2014) as well as the whole genome sequencing (WGS) approach has also been used for comparative studies (Jung *et al.*, 2012).

Many commercially important traits, such as flowering time, fall under regions in the genome where genetic differences can be quantified and it is hypothesized that the genetic difference is related to the given trait and thus, known as quantitative traits (QTL) (Salazar *et al.*, 2013). By quantifying the genetic variation, the trait of interest can be analyzed. Hence, the increased interest in the construction of linkage maps using molecular markers to identify these genetic variations, linking them to a phenotype, which can be quantified, to hypothesize the locations of genes of interest. A number of researchers have constructed genetic maps for *Fragaria* (Sargent *et al.*, 2003, 2004*a*, 2006*a*, 2007, 2008; Hadonou *et al.*, 2004; Nier *et al.*, 2006; Govan *et al.*, 2008; Zorrilla-fontanesi *et al.*, 2011; Illa *et al.*, 2011; Mahoney *et al.*, 2016) thus, providing available resources for experimenting and breeding companies.

Since the commercial strawberry is an octoploid species, the diploid woodland strawberry has been used as an alternative to studying genes in strawberry. In the past, many maps have used various PCR based markers (Ashley *et al.*, 2003; Sargent *et al.*, 2003, 2004*a*, 2006*b*, 2007, 2011; Nier *et al.*, 2006; Rousseau-Gueutin *et al.*, 2008) and these were later used to anchor the reference genome

(Shulaev *et al.*, 2011). With the advancement of technology, high-throughput maps have been generated using next generation sequencing methods (Bassil *et al.*, 2015; Sargent *et al.*, 2016).

Flowering and runnering were thought to be inversely controlled by a single locus however, Brown and Wareign (1965) showed through simple genetics, that two separate loci, then called seasonal flowering locus (SFL) and runnering locus (R) controlled the processes (Brown and Wareing, 1965; Hytönen and Elomaa, 2011). The *FvTFL1* gene characterized by Koskela, *et al.* (2012) co-localizes with the SFL and *FvTFL1* was characterized to be a strong floral repressor that maintains the vegetative phase of the plant and integrates environmental signals to maintain the identity of the meristem.

Homologs of *Arabidopsis* photoperiodic pathway genes are present in woodland strawberry genome (Mouhu *et al.*, 2009). However, the same genes play different roles in the different genotypes (Mouhu *et al.*, 2013). Recent research has shown that the photoperiodic pathway (*FvFT1- FvSOC1- FvTFL1*) is intact in the seasonal flowering genotype through LDs; although, the same pathway results in varying flowering phenotypes for the perpetual flowering mutant (Mouhu *et al.*, 2013; Rantanen *et al.*, 2015; Koskela *et al.*, 2016). In the SD seasonal flowering accession *Fragaria vesca* (FIN56), under LD, FvFT1 activates FvSOC1 which in turn upregulates *FvTFL1* inhibiting flowering. Under cool temperatures, *FvTFL1* is repressed irrespective of the photoperiod by an unknown factor and thus flowering is induced, while at 14-18°C, SD is required to downregulate *FvTFL1* and induce flowering. At higher temperatures flowering is suppressed irrespective of photoperiod because *FvTFL1* is upregulated (Rantanen *et al.*, 2015; Koskela *et al.*, 2016). Recent reports discussed that in a perpetual flowering accession *F. vesca* f. *semperflorens* Hawaii (H4), LD also promotes *FvFT1* which in turn upregulates *FvSOC1,* but due the presence

of a nonfunctional *FvTFL1*, both *FvFT1* and *FvSOC1* promote flowering (Rantanen *et al.*, 2014; Koskela *et al.*, 2016).

Research has also shown that in the SD seasonal flowering background overexpression of *FvSOC1* was able to manipulate the vegetative characteristics such as continuous runnering behavior, while silencing *FvSOC1* reduced runner formation, independently of the photoperiod. Previous studies have shown that *FvSOC1*, a homolog of the *Arabidopsis SOC1,* regulates the expression of several GA biosynthetic and signaling genes (Mouhu *et al.*, 2013).

GA is involved in cell growth and development through cell division and elongation (Mutasa-Göttgens and Hedden, 2009) and has previously been reported to be regulated by photoperiod (Hytönen *et al.*, 2009). The GA pathway is made up of complex regulation of the biosynthesis and deactivation of GA through multiple steps. Recently through fine mapping studies in Yellow Wonder (YW), a *Fragaria vesca* mutant, a deletion in the active site of *FvGA20ox4* a gene encoding GA biosynthetic enzyme, was found and this gene was identified as a likely candidate of R. The mutation gives rise to dormant shoots or shoots bearing inflorescence but not runners, which can be reversed by the application of GA (Tenreira *et al.*, 2017).

Recent ENU mutagenesis screen of the YW plants have found a mutant that regains the runnering ability and was called suppressor of runnerless (srl). Using bulk segregate mapping-by-sequencing of the F2 population, the causal gene was mapped at the end of LG4. A putative DELLA gene from the *F. vesca* genome, previously named as *FveRGA1* (Kang *et al.*, 2013), was identified as a candidate gene. This gene exhibited a nonsynonymous mutation close to a stop codon in the *srl* mutant (Caruana *et al.*, 2017).

2 OBJECTIVES

Strawberry is an economically important crop species, and with the changing climate, it has become more important to understand the environmental control of strawberry development at the genetic and molecular level. This thesis was designed to analyze the roles of known flowering genes in the control of the balance between vegetative and generative reproduction in strawberry using woodland strawberry as a model. It covers aspects of molecular genetics, plant transformation, plant architecture, phenotyping under different temperatures and photoperiods, marker development and genetic mapping. The thesis is based on three publications that address following specific aims:

I. To investigate the environmental and molecular control of the balance between vegetative and generative development in the seasonal flowering woodland strawberry.

II. To study the role of *FvCO* in the control of flowering and vegetative development in woodland strawberry.

III. To identify candidate genes involved in the control of vegetative and generative development through QTL mapping in order to facilitate the selection and breeding of new varieties of strawberry that will exploit early and late season dips in traditional strawberry production, where the market price is higher.

3 Materials and methodology

Table 1 summaries the methods utilized in the thesis. Detailed methodology is explained in the respective publications. Methods used by co-authors are in parenthesis.

Table 1: List of methods used in this thesis

Methodology	Publication
Bioinformatic analysis	(II)
cDNA synthesis	II, (II)
Crossing populations	(III)
DNA extraction	(III)
Gateway™ vector construction	(I), (II)
Genetic mapping	III
Genetic transformation	(I), (II)
Genotyping-by-sequencing analysis	(III)
Growth experiments	I, II
Marker design	III
Plant architecture	I, II
Phylogenetic analysis	(II)
RT-qPCR	II, (II)
Shoot architecture	I, II

3.1 Plant materials and experimental conditions

Two separate experiments were constructed using a seasonal flowering accession and a perpetual flowering accession. The wild F. *vesca* accession 'Punkaharju' (National Clonal Germplasm Repository accession PI551792, abbreviated as FIN56) is a seasonal flowering SD accession, which has been used in prior investigation and thus, had available transgenic lines. The F. *vesca* (L.) var. *semperflorens* (Duch.) Staudt 'Hawaii-4' ('H4') is a perpetual flowering LD plant, which had also been used in previous studies and therefore, seeds were available for *FvCO* transgenic lines that were characterized in this thesis. Table 2 and 3 summaries the plant material used and past publications in which these were introduced.

Table 2: The lines used in the publication I.

Genotypes	Line	Publication
FIN56	WT	PI551792
NOR1	WT	(Heide and Sønsteby, 2007)
SOC1-OX	*FvSOC1*-OX7	(Mouhu *et al.*, 2013)
	FvSOC1-OX11	(Mouhu *et al.*, 2013)
	FvSOC1-OX12	(Mouhu *et al.*, 2013)
SOC1-RNAi	*FvSOC1* RNAi3	(Mouhu *et al.*, 2013)
TFL1-OX	*FvTFL1*-OX2	Unpublished data
	FvTFL1-OX3	Unpublished data

FIN56 and its transformants were used to study the effects of varying photoperiod and temperature on the vegetative and generative pattern in strawberry (Table 2). H4 and *FvCO* RNAi and overexpression lines were used to study the role of *FvCO* in the control of generative and vegetative development in diploid perpetual flowering strawberry.

For the FIN56 experiment, young runner cuttings from mother plants grown in non-inductive conditions were propagated and kept in the LD greenhouse for two weeks before being moved to the growth room for a further 1.5 weeks to acclimate to the different condition prior to the start of the environmental treatments. During the environmental treatments, plants were subjected to three different temperatures (low =10 °C, intermediate = 18 °C high =22 °C) in two photoperiods (SD = 12hr, LD = 16 hr). The details of the experimental layout is elaborated in (I).

For the H4 experiment plants were grown from seeds, which were germinated on petri dishes and GFP+ plants were potted in the greenhouse under LD. At 2-3 leaf stage, the plants were moved to the growth room and allowed to acclimatize for a further 1.5 weeks before the start of the treatments. Both treatments had the same temperature of about 18 °C under different photoperiods (SD = 12hr, LD = 16 hr). Details of the experimental design are described in (II).

For (III), an available H4 × FIN56 crossing population, which was previously used for map-based cloning of *FvTFL1* (Koskela *et al.*, 2012), was used to explore QTLs linked to flowering and vegetative traits. However, only a subset of the population was used consisting of 335 seedlings of various flowering time.

3.2 Plant architecture

During and after the environmental treatments, newly opened leaves were marked weekly with a number to trace the fate of the axillary bud in each leaf axil. For the perpetual flowering H4, plants were broken down after the flowering date was recorded and the leaf axil that produced a runner was marked with “R”, those that formed branch crowns were marked as “BC” while those that produced the inflorescence were marked as “F”. Some leaf axils remained dormant and these were marked as “0”. For the seasonal flowering plants, the fate of the axillary buds was observed every

1-2 weeks by marking the newly opened leaves and the fate of the axillary buds were recorded similar to H4 plants.

3.3 Genotyping and mapping

Additional genes, to the known and studied genes were mined using genetic mapping in (III). A number of previously published SSR were used to form a basic map which was filled with segregating SNPs designed to flank the QTL regions and finally, 186 seedlings were used for high-density mapping using genotyping-by-sequencing (GBS) analysis. The details of the markers used and the analysis are described in (III).

3.4 cDNA synthesis and RT qPCR

For (I) and (II) RNA was extracted as described by (Mouhu *et al.*, 2009) and then treated with rDNAse (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturers protocol. cDNA was synthesized followed by real-time quantitative PCR (RT-qPCR), details of which can be found in (I) and (II). The reference gene used was MULTICOPY SUPRESSOR OF IRA1 (MSI1) and the calculations were done using ΔΔCt method (Pfaffl, 2001).

4 RESULTS AND DISCUSSION

4.1 Control of meristem fate in woodland strawberry (I)

Strawberry is a perennial rosette plant that can reproduce both sexually though seeds and asexually through runners that produce daughter clones. In the vegetative state, SAM produces new leaves and one AXM in the axil of each leaf. The AXM can either remain dormant or form either runners or branch crowns. After floral induction, SAM of both the main and branch crowns produces a determinate inflorescence and the total number of inflorescence per plant depends on the number of branch crowns. Since a single meristem can differentiate into one structure, there is a trade-off as to which structure is formed and this is regulated through a complex interaction between the developmental, environmental and genetic components (Guttridge, 1960; Hytönen and Elomaa, 2011). The ability to manipulate this balance would be an important tool for growers who would like to either improve flowering and consequently yield or increase the efficiency of clonal propagation.

4.1.1 Environmental regulation of AXM fate (I)

We studied environmental regulation of axillary bud differentiation in a diploid woodland strawberry that is used as a model for the cultivated strawberry, which has a more complex octoploid genome. To investigate the response of AXM to different temperatures and photoperiods, a seasonal flowering accession FIN56 was grown at three different temperatures of 10°C, 16 °C and 22°C under SD and LD (I).

At cool temperature, AXMs were insensitive to photoperiod, actively initiating branch crown formation and 100% of the plants flowered under SD, while 73.3% flowered under LDs. At intermediate temperature, however, AXM differentiation was photoperiodically regulated, where

LDs favored runner production, whereas flower inductive SDs promoted branch crown formation. About 66.7% plants flowered under SDs and 20% of the plants under LDs also flowered. Since flowering is known to affect branch crown formation, we compared flowering and non-flowering plants in both SD and LD. Under SDs, the plants that flowered had more branch crowns as compared to runners, while under LDs plants that flowered had about the same number of runners and branch crowns (Figure 3). Those that did not flower, actively produced runners over branch crowns irrespective of the photoperiod. This showed a connection between photoperiodic and developmental regulation, where runner production was favored under LDs, while branch crowns were abundant under SDs in flowering plants; however, non-flowering plants produced runners over branch crowns.

At warm temperature, AXM promoted runner formation and branch crown production was significantly reduced. Flowering was inhibited, similar to previous studies by Rantanen *et al.* (2015), who showed that high temperature upregulated *FvTFL1* expression independently of photoperiod to repress flowering. The floral inhibition caused by *FvTFL1* is the likely reason for the AXM to differentiate into runners instead of branch crowns. However, an unknown factor increases *FvTFL1* under SD at high temperature.

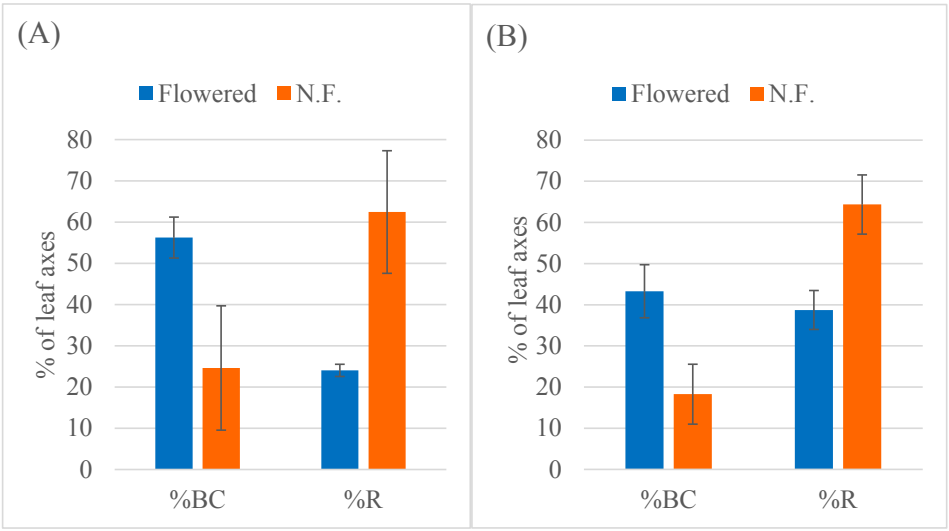

Figure 3 The effect of flowering on AXM differentiation in FIN56 in (A) SD (N = 5-10) and (B) LD (N= 6- 9) at intermediate temperature (16 °C). The average percentage of AXM that differentiated into either branch crown (BC) or into runners (R) is shown. Error bars represent standard errors, N = 5-10. It is to be noted that some AXM also remained dormant.

Studies in FIN56, could not clearly separate the environmental and developmental regulation of AXM differentiation. Therefore, genotypes that were vegetative under these conditions were used in the study. These included plants overexpressing *FvTFL1*, which is a strong floral repressor involved in both photoperiodic and thermal regulation of flowering in woodland strawberry (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2015) and NOR1 (Heide and Sønsteby, 2007), a Norwegian accession that requires obligatory vernalization prior to flowering due to the high expression of *FvTFL1* mRNA (Koskela *et al.*, 2017).

At intermediate temperature the *TFL1* overexpressing lines and NOR1 demonstrated a photoperiodic response in AXM differentiation. Similar to FIN56, LDs promoted runnering, while

SDs resulted in an increase in branch crown formation. Temperature also regulated the differentiation of AXM. The *TFL1*-OX and NOR1 plants behaved similar to FIN56 that is by increasing branch crown production at cool temperature and favoring runner production at warm temperature. In summary, *FvTFL1* has little or no direct role in the differentiation of AXM into runners in the conditions tested in this study, which is in agreement with previous reports (Koskela *et al.*, 2012, 2016). Taken together, this illustrates that environmental conditions controlled axillary bud differentiation independently of flowering. Moreover, more studies concentrating on temperature control of AXM differentiation are needed to make a definitive conclusion.

4.1.2 Genetic control of AXM fate (I)

FT1 activates the MADS-box transcription factor, *SOC1* that functions as a floral activator in both SD and LD plants (Menzel *et al.*, 1996; Lee *et al.*, 2000, 2004). Previous research illustrated that in woodland strawberry under LDs, *FvFT1* activates *FvSOC1* that in turn activates *FvTFL1* to inhibit flowering in FIN56 (Figure 3). In addition to that, *FvSOC1* promotes runner formation through the GA pathway (Mouhu *et al.*, 2013). In this study, the role of *FvSOC1* in AXM differentiation under different conditions was tested using previously generated transgenic lines. *FvSOC1* overexpressing lines showed a quantitative preference to runner production, where a higher *FvSOC1* transgene level increased the frequency of AXM differentiation into runners as compared to weaker expressing lines. The strongest overexpression line, *FvSOC1*-OX12 produced runners at all temperatures and no branch crowns were observed in this line. An increase in *SOC1* levels resulted in all the lines to become insensitive to photoperiods and abundantly produce runners except at cool temperatures, where some branch crowns were observed in the weaker *SOC1*-OX lines. Previous studies reported that at cool temperature and SD, *FvSOC1* and *FvTFL1*

expression levels in FIN56 drop (Koskela *et al.*, 2012; Mouhu *et al.*, 2013) and similar observations were found in cultivated strawberry (Nakano *et al.*, 2015; Koskela *et al.*, 2016). This indicates that the runner production in strawberries is dependent on *SOC1* levels in different conditions and that SD and cool temperature are indications of the forthcoming winter to initiate the AXM differentiation into branch crowns instead of runners and to prepare the plant to flower the following spring.

Silencing the *SOC1* expression on the other hand, did not completely abolish runner formation but the RNAi lines stopped runnering earlier than FIN56 as observed also by Mouhu *et al.* (2013). This indicates that there is a parallel pathway that is likely promoting AXM differentiation into runners independently of *FvSOC1*. This study and also Rantanen *et al* (2015) showed that in the *FvSOC1* RNAi lines, flowering was not observed at high temperatures. Taken together, it is hypothesized that an unknown factor inhibits flowering by increasing *FvTFL1* mRNA level and promotes runner formation under SD at high temperature independently of *SOC1*. More research needs to be done to find out how this temperature is perceived, how the signal is integrated and how the genes are regulated based on this information.

Hytönen *et al*. (2009) illustrated that GA promoted AXM to form runner in cultivated strawberry and Mouhu *et al.* (2013) showed the involvement of *FvSOC1* in the activation of the GA pathway genes in the woodland strawberry including a number of GA20ox. Recent studies fine-mapped the R-locus and identified a mutation in the candidate gene *FvGA20ox4* that made the enzyme inactive. Expression analysis detected that the gene was expressed in the AXM and in developing runners (Tenreira *et al.*, 2017).

Recently a runnering mutant was identified from the runnerless YW accession through chemical mutagenesis and was called *suppressor of runnerless* (*srl*). A DELLA gene named *FveRGA1* (Kang

et al., 2013) was identified as a candidate gene through QTL mapping using the M2 population. It was shown that the *srl* mutant occurred due to a nonsense mutation in the DELLA gene that enabled runnering in runnerless YW, showing that *FveRGA1* regulates AXM differentiation in strawberry (Caruana *et al.*, 2017). The DELLA proteins are repressors of GA-responsive growth, that are degraded by the E3 ubiquitin ligase in response to GA binding to GID1 receptors (Harberd *et al.*, 2009; Sun, 2010; Fukazawa *et al.*, 2015). These results prove that GA is needed for AXMs to differentiate into runners and a simplified flow diagram is illustrated in Figure 4 below.

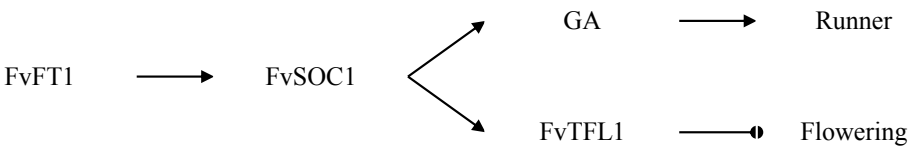

Figure 4 Role of SOC1 in the control of flowering and AXM differentiation under LDs in the woodland strawberry. The arrows indicate activation, while the arrow head with a broken circle indicates repression. FvFT1 activates *FvSOC1* which influences the fate of the AXM through the GA pathway to form runners and also controls flowering through *FvTFL1*.

The morphology of the commercial octoploid strawberry is similar to that of the diploid woodland strawberry. There is a lot of research done on many different cultivars (Gaston *et al.*, 2013; Heide *et al.*, 2013; Koskela *et al.*, 2016) that has revealed cultivar-specific thresholds controlling the physiological and developmental response to environmental changes. Like in the woodland strawberry, seasonal flowering cultivars of the cultivated strawberry are induced to flower in SDs or at cool temperatures, whereas high temperature inhibit flowering (Darrow and Waldo, 1932; Waldo and Darrow, 1932; Ito and Saito, 1962; Darrow, 1966; Bradford *et al.*, 2010; Durner, 2015).

The control of AXM differentiation in the cultivated strawberry is also similar to the woodland strawberry, where branch crown formation is promoted in floral inductive conditions whereas runnering is antagonistic to branch crown formation (Heide, 1977; Konsin *et al.*, 2001). Thus, the knowledge gained from the studies in the woodland strawberry can be applied to that of the commercial strawberry.

4.2 Photoperiodic control of meristem fate in perpetual flowering woodland strawberry (II)

4.2.1 FvCO is the only Group Ia COL protein in woodland strawberry (II)

The photoperiodic pathway revolves around the LD activation of FT protein in the leaves by a stable CO protein. This CO-FT module is conserved in many plants however, the outcome differs between SD and LD species (Hayama *et al.*, 2003). Under LDs in the facultative LD *Arabidopsis*, CO activates FT to induce flowering. However, in rice a SD plant, CO represses flowering under LDs (Hayama *et al.*, 2004).

Since photoperiod regulates flowering and also AXM in strawberry (I), this study was conducted to understand the role of *CO* in the photoperiodic pathway of the woodland strawberry. Phylogenetic analysis of the *FvCO*-like sequences found in the *F. vesca* whole- genome v1.1 assembly (Shulaev *et al.*, 2011) revealed only a single amino acid sequence that grouped together Group Ia COL proteins. This protein was previously named FvCO (Shulaev *et al.*, 2011).

4.2.2 *FvCO* controls flowering phenotype (II)

FvCO overexpressing and RNAi transgenic lines were generated in the LD accession Hawaii-4 (PI551572; National Clonal Germplasm Repository, Corvallis, OR; called H4 hereafter). H4 is a perpetual flowering mutant that has a two bp deletion in the first axon of the *FvTFL1* gene that results in a non-functional *FvTFL1* protein and thus, abolishes the SD requirement to flower. In this accession, *FvFT1* and *FvSOC1* promote flowering under LDs, whereas in the SD accessions, flowering is inhibited under LDs because *FvTFL1* is upregulated by *FvFT1* through *FvSOC1*.

The AXM differentiation into either runners or branch crowns as well as the flowering time was investigated in the H4 (WT) and in *FvCO* and *FvFT1* transgenic lines under LD and SD at 20–22 °C. H4 produced more runners under SDs than under LDs. The balance between runner production and branch crown formation was photoperiodically regulated also in the *FvCO*-OX lines. SDs promoted runner formation, but a higher percentage of branch crowns was observed in the overexpression lines than in H4 especially under LDs. Despite the difference in AXM differentiation under SD and LD, flowering was observed under both photoperiods for the overexpressing lines, although slightly later under SDs than LDs. When moved to floral inductive LD conditions, H4 continued runner formation for a longer period of time and thus produced more runners than *FvCO*-OX. The overexpressing lines flowered earlier than H4 and the average number of inflorescences produced was also higher in the overexpressing lines than in H4. Similarly, overexpressing *FvFT1* caused extreme early flowering (Rantanen *et al.*, 2015) and no runners were observed in *FvFT1*-ox plants (T. Hytönen, personal communication).

FvCO RNAi-silenced lines were insensitive to photoperiod. They formed more runners in both photoperiods and only a few branch crowns, flowered significantly later especially in LDs, and produced fewer inflorescences as compared to H4 (II), similar to the previously studied *FvFT1* RNAi lines (Koskela *et al.*, 2012; Rantanen *et al.*, 2014).

Our results (II) coincided with previous results and showed that silencing either *FvCO* or *FvFT1* delayed flowering, while overexpressing either *FvCO* or *FvFT1* (Rantanen *et al.*, 2014) advanced flowering suggesting that *FvCO* regulates *FvFT1*. Gene expression analysis of *FvCO* transgenic lines confirmed that *FvCO* activates *FvFT1* especially under LD conditions, and previous research showed that the highest *FvFT1* mRNA level was observed at intermediate temperature at 16 °C as compared to 13 and 23 °C (Rantanen *et al.*, 2015). The expression levels of the genes downstream

of *FvFT1* such as *FvSOC1* and *FvAP1* correlated with the flowering data. In comparison to the H4, *FvCO*-OX lines showed an upregulation in the *FvSOC1* and *FvAP1* expression, while the RNAi lines had reduced expression of *FvSOC1* and untraceable amounts of *FvAP1* mRNA under both photoperiods.

In H4, *FvSOC1* mRNA level negatively correlated with runner production unlike in the SD accession (I). Therefore, it is proposed that in H4, AXM differentiation is developmentally regulated through floral induction and *FvSOC1* has a minor role in the process. Upon floral transition the uppermost AXMs differentiate into new branch crowns, providing new meristems for inflorescence formation and hence, indirectly reduces runner formation (Hytönen *et al.*, 2004). However, *FvFA20ox4* that is activated by *FvSOC1,* at least in the leaves, is also needed for runner formation in perpetual flowering accessions of the woodland strawberry (Mouhu *et al.*, 2013; Tenreira *et al.*, 2017). Recent studies have reported that due to a deletion in the active site of *FvFA20ox4*, a gibberellin (GA) 20-oxidase (GA20ox) encoding gene, results in a runner-less phenotype. The mutation inhibits the production of runners and makes the AXM either dormant or form branch crowns. Another possibility is that similar to the SD accession FIN56, high temperature (22 °C) that was used in the experiments could have prevented flowering and promoted runner formation in H4 in SDs independently of *FvSOC1*. Therefore, further studies at a lower temperature is required to understand the photoperiodic regulation of the balance between vegetative and generative development in H4.

4.2.3 The photoperioidic rhythm (II)

In *Arabidopsis*, the external coincidence model suggests that *CO* expression peaks during the evening and under LDs this peak coincides with and external factor; i.e. light, resulting in the

activation of FT (Suárez-López *et al.*, 2001). To understand the photoperiodic control of *FvCO* and *FvFT1*, their expression patterns were studied in a 24 hr period. *FvCO* showed a single peak at dawn under both photoperiods in both the perpetual flowering LD (H4) and the seasonal flowering SD (FIN56) accessions. However, two peaks were observed in the *FvFT1* expression (Koskela *et al.*, 2012) and only the morning peak coincided with *FvCO* expression.

In the *FvCO*-OX lines under LDs, the diurnal expression of *FvFT1* was disrupted and the expression of *FvFT1* was always higher than that of H4 WT from 0 to 16 hrs after dawn while under SDs, *FvFT1* mRNA peaked twice at 4 and 12 hrs after dawn. The *FvCO* RNAi plants had low or undetectable *FvFT1* expression levels throughout the cycle. This shows that although *FvFT1* rhythm does not overlap with that of *FvCO*, functional *FvCO* is needed to activate *FvFT1* at both time points. Although the second *FvFT1* peak was slightly higher than the first in H4; however, the first peak was slightly higher in FIN56, the rhythm was similar in both accessions.

Previous studies demonstrated that the height of the peaks in strawberry is dependent on the light conditions (Rantanen *et al.*, 2014). Thus, darkness experiments were conducted that revealed that the *FvCO* peaks in darkness and that the light regulates its expression. Interestingly, the downregulation of *FvCO* by light at dawn is similar to that shown in the SD plant *Chenopodium rubrum* (Drabešová *et al.*, 2014). However, it was also observed that *FvCO* peak is at a different phase than the *CO* peak in *Arabidopsis,* and the dawn phase suggests similarities to other Group 1a COL genes showing a convergent evolutionary model. However, more research is needed to make any definite conclusions.

4.3 Environmental influence on the fate of meristems is an interplay between several QTL (III)

Exploring out-of-season production is an economically important topic for plant breeders. Previous mapping studies have concentrated on perpetual flowering varieties in both the octoploid, *Fragaria×ananassa Duch* and the diploid, F. vesca (Weebadde *et al.*, 2008; Iwata *et al.*, 2012; Koskela *et al.*, 2012; Gaston *et al.*, 2013; Castro *et al.*, 2015; Perrotte *et al.*, 2016); however, early flowering in strawberry has not been given much attention. One of the ways to investigate unexplored areas on genomes, is through genetic mapping (Ehrenreich *et al.*, 2009; Fan *et al.*, 2010; Sadok *et al.*, 2013; Zhang *et al.*, 2013; Bielenberg *et al.*, 2015), and recent development in molecular markers and bioinformatics tools has enabled efficient fine mapping (Bassil *et al.*, 2015; Mahoney *et al.*, 2016).

After successfully finding and functionally characterizing *FvTFL1* in an F2 cross between the perpetual flowering accession, *F. vesca f. semperflorens* 'Hawaii-4' (H4) and the seasonal flowering accession *F. vesca* subsp. *vesca* (FV) (denoted H4×FV) (Koskela *et al.*, 2012), the same mapping population was further analyzed to search for QTLs influencing flowering time and axillary bud differentiation. However, only plants which possessed at least one allele from the FV parent at the *FvTFL1* locus were included because homozygous for "H4" at that locus produced perpetual flowering phenotypes. SSR and SNP markers were developed and used for initial mapping. The map was then saturated by genotyping by sequencing (GBS)-derived SNP markers and the resultant map proved reliable when compared with the physical positions of markers from *Fvb* genome assembly (Tennessen *et al.*, 2014).

To show that the QTLs found were robust, data of selected individuals was analyzed under three different environments: a field experiment, a greenhouse experiment, in which the plants were

induced to flower in the field followed by phenotypic observations in the greenhouse and a growth chamber experiment. Based on the observations from the greenhouse experiment, a subset of 16 extremely early and 16 extremely late lines were selected for the growth chamber experiment. Several overlapping additive QTLs on linkage groups (LG), 4, 6 and 7 were found for flowering time in woodland strawberry. However, the field experiment only revealed a QTL on LG4. The QTL on LG6 mapped to the *FvTFL1* region (Koskela *et al.*, 2012) while one QTL was mapped close to the previously identified *PFRU* locus on LG4 (Gaston *et al.*, 2013; Castro *et al.*, 2015; Honjo *et al.*, 2016).

Based on the QTL on LG4 that was found close to the bx083 maker in both the greenhouse and field experiments and has previously been identified as one of the markers close to the *PFRU* locus (Perrotte *et al.*, 2016), we propose another role for *PFRU* in controlling early flowering trait in woodland strawberry. However, the other QTL upstream of *PFRU* on LG4 was observed only in specific environments.

The study discovered previously undocumented QTLs located near the marker BFaCT044 on LG7 linked to flowering, which is probably environmentally controlled as they were only detected in the greenhouse and growth chamber experiments but not the field. Alleles at the QTLs on LG4 and LG7 were analyzed and showed additive effects on flowering time and revealed that the "H4" was dominant and even a single allele from that parent delayed flowering response while homozygous "FV" alleles at *FvTFL1* also caused delayed flowering phenotype.

Previous studies have proposed two candidate genes, *FvFT2* and *FvCDF2*, for the *PFRU* locus, which are regulators of photoperiodic flowering (Perrotte *et al.*, 2016). CDFs in *Arabidopsis* are known to repress *CO* and *FT* during the morning (Song *et al.*, 2012). However, in *Fragaria*, *FvFT2* is mostly expressed in flowers and fruits (Kang *et al.*, 2013).

A candidate for the QTL on LG7 was identified as *FvELF6*, which in *Arabidopsis* has an epigenetic function of regulating chromatin through histone demethylation (Jiang *et al.*, 2007; Jeong *et al.*, 2009). This candidate seems plausible since chromatin regulation through methylation is a known function in flowering control (Jeong *et al.*, 2015) and a QTL on LG2 that co-localizes with ELF6 in almond has been shown to be related to heat requirement for flowering (Sánchez-Pérez *et al.*, 2012).

TFL1/CEN has been shown to act as a floral repressors in many species (Fan, 2010; Romeu *et al.*, 2014) including *Fragaria* (Koskela *et al.*, 2012). In this study two QTLs have been linked to that gene class, one on LG6, which has been functionally validated by Koskela *et al*. (2012) and another peak on LG7 which co-locolized with *FvCEN1*.

In addition to that, two QTLs on LG4 and LG5 indirectly affected the yield by controlling branch crown and runner production. The QTL on LG4 co-localized with the previously published *PFRU* locus (Gaston *et al.*, 2013) and was downstream of the flowering time QTL from this study. It can be hypothesized that the two QTLs on LG4 may control AXM differentiation into runners or branch crowns and flowering. However, further research is needed to derive conclusions.

A *TCP* transcription factor, *FvTCP7* (Wei *et al.*, 2016) was located close to the LG4 marker and identified as a potential candidate. Previous research has revealed that *FvTCP7* is similar to the *Arabidopsis TCP14* and *TCP15*, which regulate cell proliferation in young internodes, developing leaves and floral tissues and the mechanism varies depending on the tissue (Kieffer *et al.*, 2011). This is an interesting candidate considering that runners and branch crowns are shoots with long or short internodes, respectively, and recent research in strawberry showed that it is highly expressed in vegetative tissues such as runners, flower buds and matured flowers (Wei *et al.*, 2016).

Two homologs of DAM and SVP that function as floral repressors in *Arabidopsis* and are associated with dormancy in the Rosaceae family (Hartmann *et al.*, 2000; Bielenberg *et al.*, 2008; Fan *et al.*, 2010; Sánchez-Pérez *et al.*, 2012) were identified near the LG5 QTL. Strawberries do not undergo true dormancy, but under SDs in autumn runner formation is reduced and chilling is required to recommence development (Heide *et al.*, 2013).

Further studies are needed to functionally characterize the candidate genes presented in this study and to reveal their roles in the control of flowering and axillary meristem differentiation. Especially the analysis on the role of *FvDAM* , *FvTCP7* and PFRU in AXM differentiation and their connections to the *FvSOC1* and GA pathway (I) (Hytönen *et al.*, 2009; Mouhu *et al.*, 2013) would increase the understanding of the pathways involved in the control of AXM differentiation and flowering.

5 CONCLUSIONS

Strawberries can reproduce both sexually and asexually through runners. Many studies have explored the genetic and molecular control of flowering in the woodland strawberry (Brown and Wareing, 1965; Albani *et al.*, 2004; Sønsteby and Heide, 2008; Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014, 2015), but less is known about the control of vegetative development (Smeets and Kronenberg, 1955; Smeets, 1982; Mouhu *et al.*, 2013; Caruana *et al.*, 2017; Tenreira *et al.*, 2017).

The role of temperature and photoperiod in the fate of AXM is summarized in Figure 5. As observed previously in the control of flowering, this study found that temperature overrides photoperiod in the control AXM fate. Cool temperature or SDs enhanced branch crown formation and induced flowering, while at warm temperature and LDs, AXMs differentiated into runners and SAM remained vegetative irrespective of photoperiod. (Heide and Sønsteby, 2007; Rantanen *et al.*, 2015). However, using plants with high *FvTFL1* expression levels, we found that the environmental regulation of AXM fate is at least partially independent of the SAM fate.

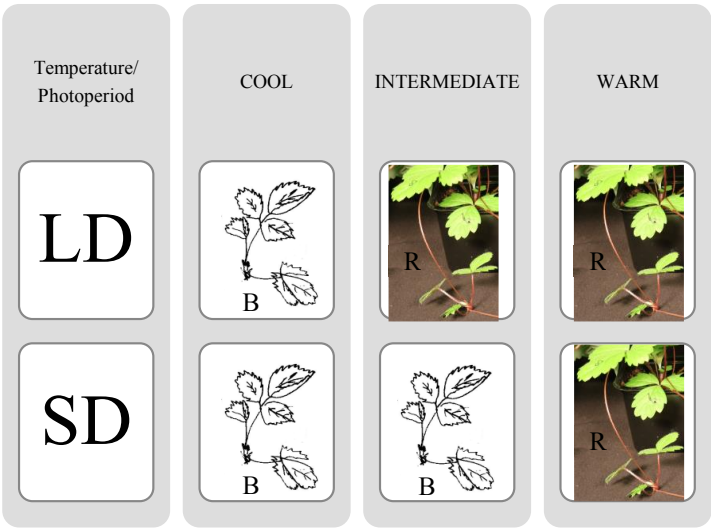

Figure 5 Interaction of photoperiod and temperature in the control of AXM differentiation. The "B" represents an increase in branch crown formation while the "R" represents an increase in runner formation at each temperature and photoperiod.

Although detailed molecular studies are needed to make firm conclusions, we propose an updated hypothetical model for the control of AXM fate in seasonal flowering woodland strawberry (Figure 6). In LD conditions, *FvCO-FvFT1* pathway is activated, and FvFT1 upregulates *FvSOC1* that has a quantitative effect on AXM fate. FvSOC1 promotes AXM to differentiate into runners by activating *FvGA20ox4* which leads to the accumulation of bioactive GA_1 and the degradation of FvRGA1 DELLA proteins. In parallel, FvSOC1 also activates *FvTFL1* to repress flowering. Under SDs, however, the photoperiodic pathway is downregulated, which changes the fates of SAM and AXM. Temperature controls flowering independently of the photoperiodic pathway by affecting the expression of *FvTFL1*, but the mechanism mediating the temperature regulation of AXM fate is unknown. Using QTL mapping, we found several QTLs related to early flowering and branch

crown formation and identified new candidate genes. Putative roles of these candidate genes in the environmental regulation of AXM and SAM fates require functional studies. Detailed knowledge on the molecular control of AXM and SAM fates is crucial for breeders to be able to develop high-yielding cultivars that can also be propagated vegetatively though runners.

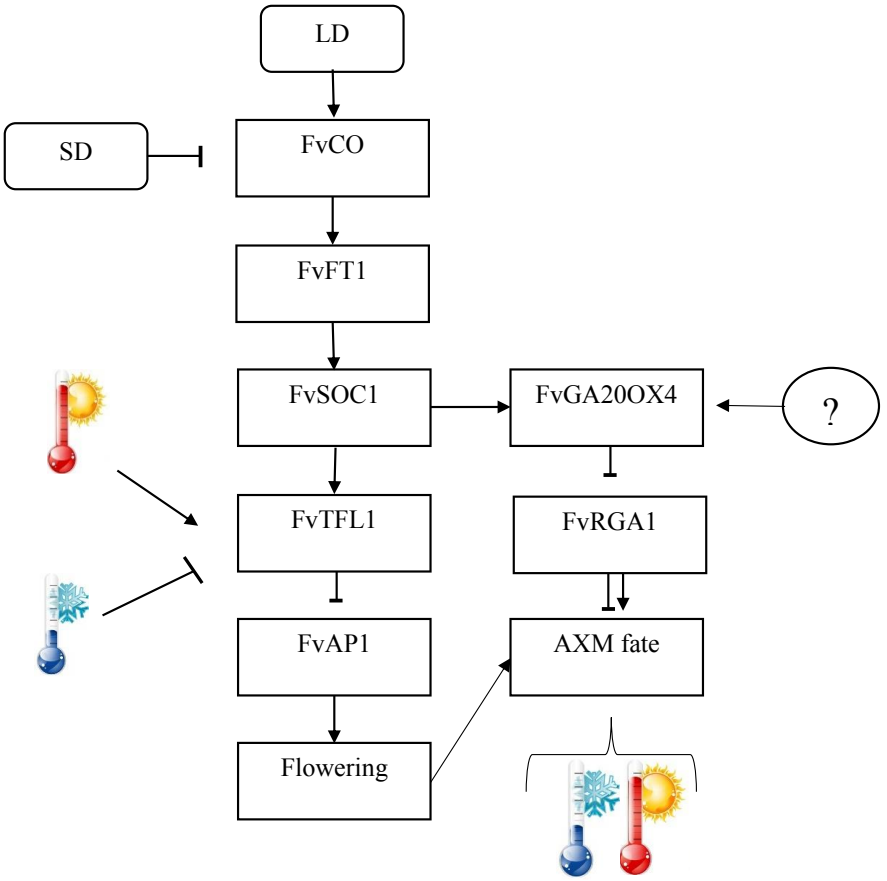

Figure 6 Model illustrating the environmental regulation of flowering and runner formation in strawberry. Arrows indicate activation and line indicate repression.

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